INTERACTION OF THE CARBOHYDRATE-BINDING PROTEIN CONCANAVALIN A WITH NORMAL AND TRANSFORMED CELLS

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Abstract.—It has been shown that the carbohydrate-binding protein concanavalin A (ConA) can agglutinate leukemic cells and cells transformed by polyoma virus, simian virus 40, chemical carcinogens, and X-irradiation. This protein did not agglutinate normal cells under the same conditions. The agglutination was reversed by competition with α-methyl-D-glucopyranoside (α-MG), a carbohydrate that strongly binds to ConA, but not by the carbohydrates α-methyl-L-fucopyranoside or N-acetylglucosamine, with no binding or weak binding to ConA. Destruction of the α-MG binding sites of the native protein by removal of bivalent metal ions abolished the agglutination produced by the native protein. The treatment of cells with trypsin resulted in the agglutination of normal cells by ConA and a decrease of agglutinability of transformed cells. When nonagglutinating untransformed 3T3 cells were infected with simian virus 40 and normal rat cells were infected with polyoma virus, the infected cells became agglutinable several days after virus infection. The percentage of cells agglutinated, about 50 per cent, was much higher than the percentage of cells hereditarily transformed. The results indicate that the surface membrane of transformed cells contains sites that interact with the α-MG binding sites of ConA, that such sites can be found on the surface membrane of normal cells after treatment with trypsin, and that the change in the surface structure from normal to transformed occurs in cells that are abortively transformed.

Agglutination of cells by substances with different binding sites should be of value in elucidating the structural changes in the surface membrane that accompany the transformation of normal cells by carcinogenic agents. A substance from wheat germ that agglutinates transformed cells has previously been described.[1, 2] Studies with this substance have indicated that this agglutinin is a glycoprotein,[2] that the agglutination can be inhibited by competition with N-acetylglucosamine,[2, 3] and that after treatment with proteases, untransformed cells can be agglutinated and contain the same or similar receptor sites as transformed cells.[4]

The present experiments were undertaken to find proteins with different binding sites that can be used for studies on the surface membrane of normal and transformed cells. It was found that when cells were dissociated with disodium versenate and not treated with trypsin, the crystalline Jack bean protein concanavalin A[5] (ConA), which binds α-methyl-D-glucopyranoside[6, 7] and various other carbohydrates, agglutinated cells transformed by viral and nonviral carcinogens, but did not agglutinate normal cells and the untransformed cell line 3T3. The present communication reports the results of these experiments with

ConA together with studies on (1) the chemical nature of the binding sites on transformed cells, (2) the difference in the structure of normal and transformed cells, and (3) the acquisition of the structure of transformed cells after infection of normal cells with polyoma virus and infection of untransformed 3T3 cells with simian virus 40.

Materials and Methods.—Cells and cell cultures: The *in vitro* transformed cells used in the present experiments consisted of lines of golden hamster, rat, and 3T3[8] cells transformed by polyoma virus; SWR mouse, 3T3, and human cells transformed by simian virus 40 (SV40); 3T3 cells doubly transformed by polyoma and SV40; and hamster cells transformed by benzo(a)pyrene,[9] after treatment with dimethylnitrosamine,[10] and by X-irradiation.[11] The lines of untransformed and virus-transformed 3T3 cells and the line of SV40-transformed human cells were kindly supplied by Drs. H. Green and G. Todaro. The leukemic cells consisted of three cultured lines of leukemias derived from an erythroid (CICPR), myeloid (P1081), and lymphoid (MDA) leukemia induced *in vivo*, as described previously.[12] The normal cells used consisted of secondary cultures of golden hamster, rat, and mouse (SWR) embryo cells, and mouse spleen and rat bone marrow cells taken directly from animals. The spleen and bone marrow cells were prepared as suspensions in phosphate buffered saline (PBS) as described.[13]

Cells were cultured in plastic (Falcon Co.) Petri dishes in Eagle's medium with a fourfold concentration of amino acids and vitamins (EM) either with 10% calf serum or with 10% horse serum for leukemic cells. All the cells, except the leukemic cells that grew in suspension, were routinely passaged in a 0.25% trypsin (1:300) solution. Secondary cultures of normal embryo cells were prepared from 4- to 5-day-old primary cultures. For agglutination experiments, cells were used 3–5 days after subculturing.

Concanavalin A: The protein, kindly supplied by Dr. A. J. Kalb, was prepared from Jack bean meal (Sigma Chemical Co.) by two crystallizations[5] and stored as a solution in saturated NaCl at room temperature. Demetallized ConA[6] was stored at −15°C.

Solutions of carbohydrates and trypsin: The carbohydrates α-methyl-D-glucopyranoside (Pfanstiehl Labs), α-methyl-L-fucopyranoside, kindly supplied by Dr. A. J. Kalb and prepared as described,[14] and N-acetylglucosamine (Chas. Pfizer and Co.) were diluted in distilled water. Crystallized and lyophilized trypsin (Worthington Biochemical Corp.) was diluted in a solution containing 0.35 gm $NaHCO_3$, 8.0 gm NaCl, 0.4 gm KCl, and 1.0 gm dextrose per 1000 ml distilled water.

Agglutination assay: Cultured cells that did not grow in suspension were washed two or three times with Ca- and Mg-free PBS, and removed from the Petri dish with a solution of 0.02%, disodium versenate in 8.0 gm NaCl, 0.2 gm KCl, 1.15 gm Na_2HPO_4, and 0.02 gm KH_2PO_4 per 1000 ml distilled water. These suspended cells and the leukemic cells that grew in suspension were washed one or three times with Ca- and Mg-free PBS and then diluted in Ca- and Mg-free PBS at a concentration of $1–3 \times 10^6$ cells per ml. To test for agglutination, 0.5 ml of different concentrations of ConA diluted in distilled water were mixed with 0.5 ml of the cell suspension in a 35-mm Petri dish (Falcon Co.). The presence and time required for agglutination was observed with a binocular microscope.

Virus infection: Untransformed 3T3 cells and secondary cultures of normal rat embryo cells were seeded at 5×10^5 cells per 50-mm Petri dish and infected with SV40 and polyoma virus, respectively, at 1 day after seeding. After 2 hr for virus adsorption, the cultures were washed once with EM, and new EM with 10% calf serum was added. The rat cells were infected with CsCl purified virus from a large plaque (LP) or small plaque (SP)[15] strain of polyoma. The 3T3 cells were infected with SV40 grown on BS-C-1 cells,[16] and control cultures were treated with an equal amount of a sonicated extract of uninfected BS-C-1 cells.

Results.—Agglutination of transformed cells by concanavalin A: Cell lines transformed by polyoma virus, SV40, two chemical carcinogens and X-irradia-

tion, and an erythroid, a myeloid, and a lymphoid leukemia were tested for agglutination by ConA. All the 14 lines tested were agglutinated by this carbohydrate-binding protein. The degree of agglutinability varied with the different cell lines. The time required for agglutination with aggregates of at least three to five cells, at different concentrations of ConA, is shown in Figures 1 and 2. The leukemic lines formed only small aggregates of about five cells each even after 30 minutes with 500 μg ConA/ml, whereas under the same conditions the aggregates in all other transformed cell lines contained about 30–100 cells. There was no agglutination of controls of these lines after 30 minutes of incubation with the same concentration of NaCl without ConA. No agglutination was observed even after 30 minutes with 500 μg ConA/ml with normal cells from hamster, rat, and mouse embryo secondary cultures, mouse spleen and rat bone marrow directly from animals, and cells from the untransformed mouse line 3T3 and the monkey line BS-C-1.

Reversibility of agglutination by competition with α-methyl-D-glucopyranoside: The three carbohydrates α-MG, α-methyl-L-fucopyranoside, and N-acetylglucosamine were tested for their ability to reverse the agglutination produced by ConA. Hamster polyoma LP-transformed cells were incubated with 500 μg ConA/ml, and aggregates of about 50 cells were formed after 15 minutes of incubation. One-ml samples of the carbohydrates were then added, and the time

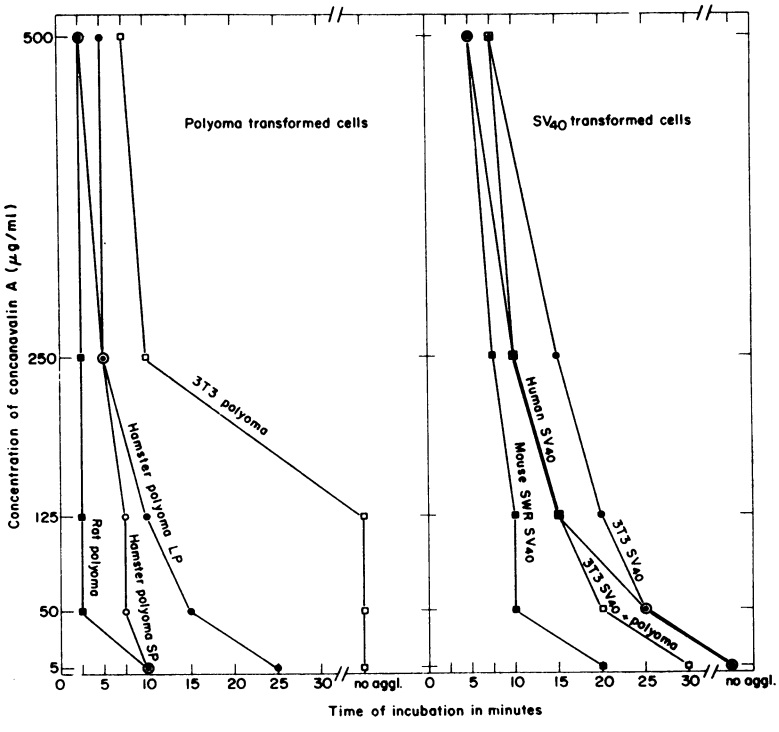

FIG. 1.—Time required for agglutination by concanavalin A of cells transformed by polyoma virus and SV40. The aggregates formed by agglutination contained at least three to five cells. *No aggl.*, no agglutination after 30 min of incubation.

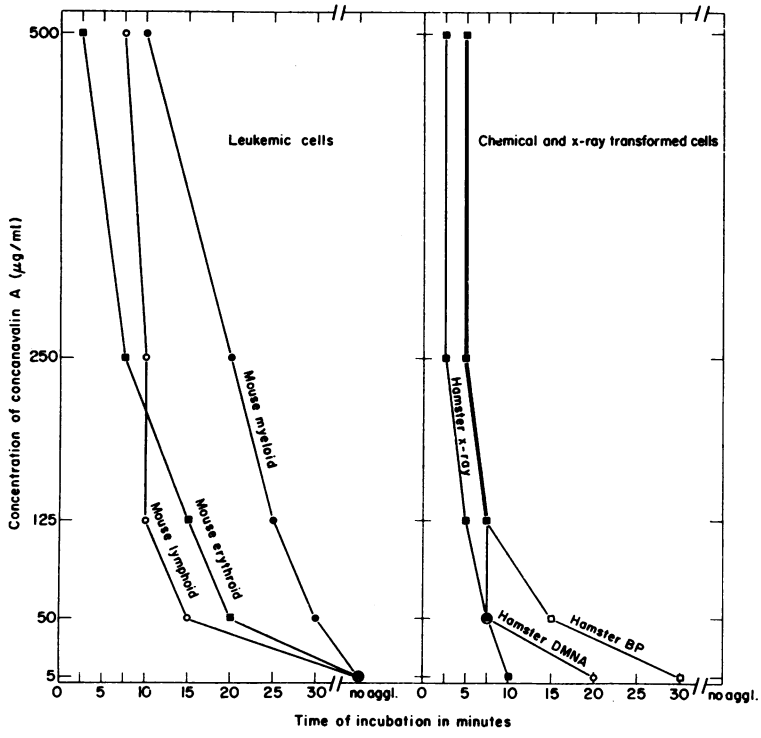

FIG. 2.—Time required for agglutination by concanavalin A of leukemic cells and cells transformed by chemical carcinogens and X-irradiation. The aggregates formed by agglutination contained at least three to five cells. *No aggl.*, no agglutination after 30 min of incubation. *BP*, benzo(a)pyrene. *DMNA*, dimethylnitrosamine.

required for dissociation of the aggregates was determined. The results indicate (Table 1) that dissociation of aggregates was obtained only by α-MG. This dissociation was almost complete, and reduced the size of the aggregates to about five cells. All the other lines of transformed cells tested also showed a dissociation of aggregates by α-MG, and the degree of this dissociation varied with different cell lines. Since the leukemic cells only formed aggregates of about five cells even at 500 μg ConA/ml, these cells could not be accurately tested for the

TABLE 1. *Reversibility of agglutination by competition with α-methyl-D-glucopyranoside.*

Incubation with carbohydrates (min)	Molar Concentrations of Carbohydrates: α-methyl-D-glucopyranoside 10^{-2}	α-methyl-D-glucopyranoside 10^{-3}	α-methyl-D-glucopyranoside 10^{-4}	α-methyl-L-fucopyranoside 10^{-2}, 10^{-3}, or 10^{-4}	N-acetyl-glucosamine 10^{-2}, 10^{-3}, or 10^{-4}
5	+	−	−	−	−
10	+	−	−	−	−
15	+	+	−	−	−
20	+	+	−	−	−
25	+	+	+	−	−
30	+	+	+	−	−

+, Almost complete dissociation of aggregates; −, no dissociation of aggregates.

dissociation of aggregates by α-MG by the method used for the other cell lines.

Inactivation of concanavalin A by demetallization: Removal of bivalent metal ions of the native protein destroys the α-MG binding sites of ConA.[6] Demetallized ConA was compared with the native protein for its ability to agglutinate hamster polyoma LP-transformed cells. The results (Table 2) indicate that destruction of the α-MG binding sites by demetallization also inactivated the agglutination produced by the native protein. The procedure used for demetallization does not remove all the bivalent metal ions.[6] This can account for the agglutination observed after 30 minutes' incubation with the highest concentration (250 μg/ml) of the demetallized ConA (Table 2).

TABLE 2. *Inactivation of concanavalin A by demetallization.*

Time of incubation (min)	Concentration (μg/ml) — Concanavalin A: 5	50	125	250	Demetallized concanavalin A: 5, 50, or 125	250
2.5	−	−	−	−	−	−
5	−	−	−	+	−	−
7.5	−	−	−	+	−	−
10	−	−	+	+	−	−
15	−	+	+	+	−	−
20	−	+	+	+	−	−
25	+	+	+	+	−	−
30	+	+	+	+	−	+

+, Agglutination with aggregates of at least three to five cells; −, no agglutination.

Effect of trypsin on the agglutination of normal, 3T3, and transformed cells: Samples of 3×10^6 hamster normal, hamster polyoma LP transformed, 3T3, and SV40-transformed 3T3 cells were incubated with 10 ml of crystallized, lyophilized trypsin solution at three concentrations for 15 minutes at 37°C. The cells were then washed three times with Ca- and Mg-free PBS and tested for agglutination with 250 μg/ml native or demetallized ConA. The results indicate (Table 3) that this treatment with trypsin resulted in a gain of agglutinability of normal and 3T3 cells with native ConA, whereas the trypsin treatment reduced the agglutinability of the transformed cells. As with the aggregates of transformed cells produced by ConA, the aggregates formed by the trypsin- treated normal and 3T3 cells were dissociated by α-MG.

TABLE 3. *Effect of trypsin on the agglutination of normal, 3T3, and transformed cells.*

Cells	Concentration of Trypsin (mg/ml) — Concanavalin A: 0	0.001	0.01	0.1	Demetallized concanavalin A: 0	0.001	0.01	0.1
3T3	−	+	++	++++	−	−	−	+
SV40-transformed 3T3	++++	++	+	−	−	−	−	−
Normal hamster	−	++	+++	++++	−	−	−	−
Polyoma-transformed hamster	++++	++++	+++	+	−	−	−	−

The density and size of aggregates were scored in a scale from − to ++++. The data for transformed cells were taken at 20 min and that for 3T3 and normal cells at 10 min after addition of native or demetallized protein. When tested at 20 min, the 3T3 and normal cells gave an agglutination of ++++ with the native protein at all the concentrations of trypsin used.

Agglutination after virus infection of normal and 3T3 cells: Normal rat embryo cells were infected with polyoma virus and 3T3 cells were infected with SV40, and the infected cells were tested daily for their ability to be agglutinated by 500 μg ConA/ml. The 3T3 and rat cells were used in these experiments, since there was no detectable synthesis of infectious virus after SV40 infection of 3T3 cells, and little or no synthesis of infectious virus after polyoma infection of normal rat cells.[17] The results obtained with 3T3 cells infected with SV40 at a virus:cell ratio of 50 plaque-forming units (PFU) per cell are shown in Table 4. The data indicate that there was no agglutination of the infected cells after virus adsorption (0 time) and at one or two days after virus infection. However, the cells started to form aggregates at three days after infection, and at seven days about 50 per cent of the cells had formed aggregates containing about 30–100 cells. These aggregates were dissociated with α-MG.

TABLE 4. *Agglutination of 3T3 cells after infection with SV40.*

	Days after virus infection							
	0	1	2	3	4	5	6	7
Agglutination	−	−	−	+	+	+	++	++++

The density and size of aggregates were scored in a scale from − to ++++.

At the seeding level used in these experiments, there were about two cell divisions during the first three days after virus infection. The cells were subcultured at seven days after virus infection, with the concentration of disodium versenate used in the agglutination assay, by seeding 5×10^5 cells per 50-mm Petri dish. When tested 3–4 days later, there was no longer any observable agglutination by ConA. The further multiplication of the infected cells induced by this subculture was thus associated with a loss of agglutinability. (Control cultures of 3T3 cells showed no agglutination by ConA at any time during the experiments.) In contrast to the about 50 per cent cells involved in agglutination at 7 days after virus infection, only about 0.5 per cent of the cells were hereditarily transformed, as measured by their ability to form colonies of transformed cells. Similar results were obtained after infection of normal rat embryo cells by the LP or SP strain of polyoma virus at a virus:cell ratio of 100 PFU per cell.

Discussion.—The present results have indicated that in the absence of trypsin treatment, the carbohydrate-binding protein ConA agglutinates transformed but not normal cells or untransformed 3T3 cells. The reversal of agglutination by competition with α-MG and the inactivation of agglutination by demetallization have indicated that agglutination is produced by ConA by the sites on the protein that are involved in carbohydrate binding. It should now be possible to isolate and chemically identify the cell surface sites that interact with ConA. It has been suggested that ConA has two binding sites for α-MG per molecule.[6] Agglutination can therefore result from the binding of the ConA molecules to two cells.

The competition experiments have indicated that agglutination by ConA was reversed by competition with α-MG, in contrast to the reversal of agglutination by the wheat germ agglutinin by competition with N-acetylglucosamine.[2, 3] This indicates that the surface of transformed cells contains different sites for interaction with ConA and wheat germ agglutinin. A decrease in the agglutina-

tion of transformed cells by ConA was found after treatment with trypsin. The sites for ConA on the cell surface can thus be removed or destroyed by treatment with this protease.

The finding that agglutination is produced in untransformed cells by ConA and by wheat germ agglutinin[4, 18] after treatment with trypsin supports the assumption that untransformed cells contain the same or similar sites as transformed cells in a cryptic form, and that these sites can be exposed after treatment with a protease.[4] The different degrees of agglutination found with the different transformed cell lines tested in the present experiments indicate that different lines of transformed cells may vary in the number of exposed sites. It is of interest that the formation of cell variants with a reversion of properties characteristic of transformed cells[19] is associated with a decrease or loss of agglutinability by ConA in the absence of protease treatment.[20] The change in cellular regulatory mechanism that is produced by transformation can be ascribed to a change in the cell surface.[21] The change in the surface structure resulting in exposure of surface sites could be the main cause of this change in regulation. The induction of substances such as the Forssman type antigen in relation to cell organization[22] could also be due to the same mechanism. The number of sites exposed on the cell surface can be directly determined by the absorption of labeled ConA.[23]

The results obtained after infection of untransformed cells with SV40 and normal cells with polyoma virus have indicated that the change in the structure from normal to transformed can be obtained in about 50 per cent of the cells several days after virus infection. The several days required after infection for the detection of agglutinability, and the increase in cell number during this period, suggest that this change in structure requires cell replication. The per cent of cells agglutinated after infection was much higher than the per cent of cells that were hereditarily transformed. It has previously been shown that a temporary change of some cellular properties can be found after virus infection in a much higher per cent of cells than those that are hereditarily transformed,[17, 24] a phenomenon that has been called abortive transformation.[24] The present results indicate that abortive transformation can result in the same change in the cell surface as that found in hereditary transformation.

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